TECHNICAL NOTE

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Paternity Investigation Among Known False Trios: ABO, Rh, MNSs, Kell, Duffy, Kidd, and HLA Systems

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ABSTRACT: This paternity study was performed with trios in which the putative father was not the biological father (NBF), in order to evaluate adjustment of genetic markers employed to disclose non biological fathers for the population, and the biological meaning of likelihood of paternity in casework.

All 923 generated trios had ABO, Rh, MNS, Kell and HLA systems tested; 372 of them also had Duffy and Kidd systems tested.

The most powerful exclusion system was HLA, followed, in this order, by ABO, Rh, Duffy, MNSs, Kidd, and Kell. Taking into account the Indian/black/white historical miscegenation background in the population, an improvement in the performance of red blood cells as disclosers of non biological fathers could be achieved, if particular additional sera were used.

In the group tested with seven different systems, direct exclusions were observed in 90.31%, and they were single system exclusions in 26.61%. In order to avoid the remote possibility of mutation, it is suggested that the number of used systems be increased. Indirect exclusions were verified in 8.87% and only 0.81% of NBF were not excluded at all. In this last group, probabilities of paternity were calculated and two values greater than 95% were obtained.

To be able to accomplish the "visum et repertum" duty and to assist the court, the expert should equally emphasize: a) the probability of paternity of the alleged father and the possibility of finding an unexcluded NBF; b) the actual performance of systems used to uncover NBF, together with the probabilities of paternity of those who were not discovered; c) the previous referenced trend of probabilities of paternity of true and of non-biological fathers to cluster in distinct class intervals of likelihood of paternity.

KEYWORDS: pathology and biology, paternity investigation, blood, red blood cell systems, HLA

Genetic markers were used to solve a paternity dispute as early as 1927, in Brazil [*1*]. Ever since, the demands have grown steadily, more sharply over the last decade.

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This growth is due to several causes, some of which could be emphasized: increased demand due to misleading mass communication on the technical advances and aggravation of economical pressures associated with more tolerant social behavior.

Incompatible exclusion results are biologically trustworthy; the compatible non exclusion ones indicate the possibility of genetic association. Tests performed should reach at least 95% of Cumulative Probability of Exclusion—CPE [*2*] by using multiple systems. With such tests, calculation of the likelihood of paternity can be performed, to evaluate the biological meaning of compatibility as a "proof" of genetic association between an alleged father and child. The extreme polymorphism of the Human Leukocyte Antigens (HLA) system allows high CPEs. If few other genetic markers are used together with HLA, erroneous conclusions can be drawn: not only is it possible to miss an incompatibility, but the false probability of paternity thence calculated may be influenced by a sole polymorphic system [*3*].

The purposes of this study were: a) to evaluate the adjustment of genetic markers used to disclose nonbiological fathers (NBFs) for the population; b) to discuss the biological meaning of likelihood of paternity in casework; c) to suggest, in the light of Brazilian legislation, what should be expert's role.

Material and Methods

Generated trios were created by displacement of the alleged father (AF) in one trio to the next trio. Trios were selected to maintain the same racial classification of the NBF and mother and child. Cases selected were in chronological order, over an 18 month period. All tests were performed upon judicial order.

Tests for red blood cells (RBC) antigens included anti-A, -B, -O; -C^w, -C, -c, -D, -E, -e; -M, -N, -S, -s; -K and -k; -Fy^a, -Fy^b; -Jk^a, -Jk^b sera. HLA specificities recognized were HLA-A1, A2, A3, A9, A10, A11, A28, A19, B5, B7, B8, B12, B13, B14, B15, B16, B17, B18, B21, B22, B27, B35, B37, B40, besides Bw4 and Bw6. HLA-A and -B sera available to identify splits varied during the period. According to others [*3*], systems used should allow a combined CPE above 95%, whether Duffy and Kidd markers were used or not.

All sera were from commercial sources: Biotest S/A Indústria e Comércio (São Paulo—Brasil), Biotest A.G. (Frankfurt—Germany) and Immucor Inc. (Norcross, Georgia) for the RBC phenotyping; Biotest A.G. (Frankfurt—Germany), Pel-Freez Clinical (Wisconsin), C-Six Diagnostics, Inc. (Wisconsin) for HLA-specificities. RBC antigens were identified by usual tube agglutination techniques following the manufacturer's instructions; HLA specificities were identified by the microlymphocytotoxicity method.

Likelihood of paternity - W [*3*] was calculated when the NBF was unexcluded. Gene frequencies used for ABO, Rh, MNS and Kell systems were obtained in the same laboratory where this study was conducted [*4*]. Other Caucasoid gene frequencies were used for Duffy and Kidd [*5*] and HLA [*6*] systems.

RBC systems exclusions were grouped into direct and indirect classes [*3*]. HLA system exclusions were considered direct when at least one obligatory paternity specificity was absent in markers identified in the NBF. All incompatibilities based on blanks in either the NBF or child (C) were considered indirect exclusions.

All generated trios (923) were analyzed in relation to exclusions of NBFs. The initial criterion to classify was verification of at least one direct exclusion. Part of the artificial trios (372), tested in 7 systems, was subsequently subgrouped with the addition of indirect exclusions. Thus, the following classes were created: excluded men not considered, when only indirect exclusions were verified; and false biologic fathers, when there were no incompatibilities between NBF and child. As to the number of loci presenting direct exclusion(s), the following groups were created, according to the absence or presence of

TABLE 1—*Interpretation of test results as direct exclusions from five systems on 923 generated trios classified according to racial background.*

System	Racial group of generated trios							
	White		Mulatto		White + Mulatto		Total	
ABO	65[a]	(14.44)[b]	28	(16.28)	55	(18.27)	148	(16.03)
Rh	58	(12.89)	31	(18.02)	40	(13.29)	129	(13.98)
MNS	51	(11.33)	22	(12.79)	35	(11.63)	108	(11.70)
Kell	14	(3.11)	6	(3.49)	7	(2.33)	27	(2.92)
HLA	366	(81.33)	141	(81.98)	242	(80.40)	749	(81.15)
Total	450		172		301		923	

[a]Number of trios.
[b]% of trios of similar racial background.

concurrent direct/indirect exclusions: single (only one direct), isolated (one direct plus one or more indirect) and multiple (more than one direct).

Results

Tables 1 and 2 show the results of direct exclusions of NBF per system according to the racial classification of generated trios. It was observed that HLA system was the most effective in detecting a NBF, followed by ABO, Rh, Duffy, MNSs, Kidd and, finally Kell.

Cumulated direct and indirect RBC &/or HLA exclusion results are presented in Table 3. It was noticed that a NBF was revealed through one or more direct exclusions in 90.32% of generated trios and, not excluded in 0.81%. Doubtful results (one or more indirect exclusions) were obtained in 8.87%.

Results related to the number of loci with direct exclusion(s) according to the presence or absence of concurrent direct/indirect exclusion(s) are presented in Table 4. It was observed that single (only one direct) exclusions occurred in 26.61% of NBF.

The NBF was not identified in 3 generated trios (Table 3). Their probabilities of paternity (RBC, HLA, RBC, and HLA) and HLA common haplotypes between each NBF/child duo, together with their respective assumed frequency [6] are presented in Table 5. The probabilities of paternity of two NBFs were over 95%.

Discussion

Bearing in mind that the only completely reliable results in actual paternity testing are direct exclusions, the NBF of generated trios were treated in the same way.

The most powerful exclusion system was HLA, corresponding to its high polymor-

TABLE 2—*Interpretation of test results as direct exclusions from two systems on 372 generated trios classified according to racial background.*

System	Racial group of generated trios							
	White		Mulatto		White + Mulatto		Total	
Duffy	16[a]	(9.52)[b]	10	(13.70)	19	(14.50)	45	(12.10)
Kidd	12	(7.14)	5	(6.85)	9	(6.87)	26	(6.99)
Total	168		73		131		372	

[a]Number of trios.
[b]% of trios of similar racial background.

TABLE 3—*Interpretation of test results from seven genetic systems on 372 generated trios classified according to racial background.*

Results of tests	Racial group of generated trio							
	White		Mulatto		White + Mulatto		Total	
Direct exclusion (RBC &/or HLA)	151	(89.88)	68	(93.15)	117	(89.31)	336	(90.32)
Indirect exclusion (RBC &/or HLA)	15	(8.93)	5	(6.85)	13	(9.92)	33	(8.87)
Not excluded	2	(1.19)	0	(0)	1	(0.76)	3	(0.81)
Total	168		73		131		372	

[a]Number of trios.
[b]% of trios of similar racial background.

phism [7]. Among RBC systems, ABO uncovered 16.03% of NBF, comparable to the figures obtained by others [3,8a], while exclusions observed in Rh, Duffy, MNSs and Kell were lower. This divergence can be attributed to the historical Negro miscegenation background of the population and probably to the strict exclusion criterium adopted. In Rh system, *e* variants [8b], mainly e^s, are not infrequent in Negroes. As the anti-e^s serum was not available for routine typing, the possible presence of e^s antigen in the generated trios had to be considered whenever antithetical sera identified single different antigens in NBF and child (reverse homozygosity). In the Duffy system, the frequent Fy allele in Negroes, with an estimated frequency between 23 to 45% [5] and now defined as Fy^4, [9] could not be identified due to lack of specific serum; hence, all incompatible phenotypes presenting only one detectable antigen were considered suspect. In MNSs sys-

TABLE 4—*Interpretation of test results from seven genetic systems on 372 generated trios classified according to racial background: number of loci with direct exclusion(s) concurrent or not with other exclusion(s).*

Number of loci	Exclusion		Racial group of generated trio							
	Direct	Indirect	White		Mulatto		White + Mulatto		Total	
Single	1	0	48[a]	(28.57)[b]	19	(26.03)	32	(24.43)	99	(26.61)
		1	31	(18.45)	9	(12.33)	25	(19.08)	65	(17.47)
Isolated	1	2	7	(4.17)	4	(5.48)	4	(3.05)	15	(4.03)
		3					1	(0.76)	1	(0.27)
	Total		38	(22.62)	13	(17.81)	30	(22.90)	81	(21.77)
	2	0	39	(23.21)	20	(27.40)	28	(21.37)	87	(23.39)
		1	15	(8.93)	8	(10.96)	8	(6.11)	31	(8.33)
		2	1	(0.59)	1	(1.37)	2	(1.53)	4	(1.07)
Multiple										
	3	0	6	(3.57)	5	(6.85)	9	(6.87)	20	(5.38)
		1	1	(0.59)	1	(1.37)	6	(4.58)	8	(2.15)
	4	0	3	(1.79)	1	(1.37)	2	(1.53)	6	(1.61)
	Total		65	(38.69)	36	(49.31)	55	(41.98)	156	(41.93)
Total			168		73		131		372	

[a]Number of trios.
[b]% of trios of similar racial background.

TABLE 5—*Probabilities of paternity of three non-biological fathers (NBF) using RBC markers, HLA(A,B) specificities and both; shared HLA haplotype between NBF/child and its frequency.*

Trio[a]	Probability of paternity (W)[b]			Shared HLA haplotype between NBF/child (frequency—%)
	RBC	HLA	RBC & HLA	
1	62.79	61.29	72.77	2,Y (0.0015)
2	56.08	95.02	96.08	11,44 (0.0014)
3	87.54	94.31	99.15	2,18 (0.0182)

[a]1 & 3: White; 2 White and Mulatto.
[b]Prior probability 0.5.

tem, the frequency of the silent allele S^{u} in Negroes is between 10.53% [*10*] and 12.29% [*8c*]; in Northeastern Brazil [*11*], its frequency was estimated at 3.27% and, in Rio de Janerio, between 4% and 11% [*5*]. Thus, all incompatible phenotypes presenting only one detectable antigen were also considered suspect. This procedure can lead to an underestimate of direct exclusions. In the Kell system, bearing in mind the historical Brazilian miscegenation, observed values could possibly be improved if anti-Js^{a} and -Js^{b} had also been used.

In the Kidd system, because of the *Jk* allele frequency in Mato Grosso Indians [*12*], the historical Indian/White miscegenation and *Jk* frequency between 7 to 9% in Rio de Janerio [*5*], all incompatible phenotypes with only one detectable antigen were consequently considered suspect, although in this study no *Jk*(a-b-) phenotypes were found. This could also result in underestimate of direct exclusions, when compared to other data [*3*].

These data suggest that efficiency of RBC test results in uncovering NBFs could possibly be improved if particular, non commercial sera, were also used.

In real disputed paternity cases, it is remotely possible that, single exclusions could be due to mutation [*8a*]. It was observed that single (only one direct) exclusions occurred in 26.61% of NBF in the group with seven tested systems. This figure suggests that an extension of the number of genetic markers tested is desirable.

The calculation of probability of paternity should be performed in actual cases if alleged father is not excluded, since "multiple systems that provide a falsely accused man with, on the average, a 95% probability of obtaining evidence of non paternity" [*2*] are used.

Assuming, subjectively, that seven systems fulfill the multiple systems requirement and, that the 95% CPE level was broadly attained (90.32% of NBF were definitely excluded and 8.87% of them would probably be uncovered by further necessary tests), W values of three nonexcluded NBF were then calculated. The results obtained were 72.77%, 96.08% and 99.15%, "formal hint of paternity," "very likely paternity" and "extremely likely paternity," respectively [*13*]. These data are not in agreement with the expected clustering of W values below 95% for NBF [*13*], probably due to an insufficient number of analyzed cases. However, it is interesting that probabilities of paternity calculated for two different HLA haplotypes with very close population frequencies lead to distinct W results. This can be explained because the population frequency of a particular genotype depends on the frequencies of its haplotypes. As a consequence, even the same haplotype may have different probabilities of transmission and W values thence calculated may be different. In the same way, taking into account all systems used, the high W values obtained for two NBFs can also be explained. In other words, the population frequency of each particular constellation of genetic markers plays a role in the calculated probability of paternity (W). It would be ideal to perform all available tests in each case, to "dilute" possible distortions of W and to eliminate

the chance of exclusion in the next system [*14*]. But there are practical restrictions to unlimited tests. To reduce possible deviations to a minimum, significant paternity W values could be displaced to upper classes. This method of managing the matter would give rise to a problem: true biological fathers who occasionally show lower W values. It is a sort of vicious circle, since the current scheme has its shortcomings. Probably, the routine utilization of DNA polymorphisms will help [*14*].

In Brazil, there is no positive legislation for presumption of paternity, using a determined arbitrary level of probability of paternity. Theoretically, W values obtained, when an alleged father is not excluded, should be considered as just another piece of evidence. However, it is a common belief that this W result alone should receive a great deal of importance when paternity-non paternity possibilities are legally considered. Thus, in every case, in order to accomplish in full the "visum et repertum" duty in strict conformity with its principle and to assist the court, the expert should equally emphasize: a) the probability of paternity of the alleged father and the possibility of finding an unexcluded NBF; b) the actual performance of the systems used to uncover NBFs together with W values of those who were not discovered; c) the previous referenced trend of probabilities of paternity of true fathers and of NBF to cluster in distinct class intervals of W [*13*].

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